[30] Carotenoid Analysis in Mutants from *Escherichia coli* Transformed with Carotenogenic Gene Cluster and *Scenedesmus obliquus* Mutant C-6D

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Introduction

Carotenoid biosynthesis in plants is well regulated, and acyclic carotene precursors of α- and β-carotene and of corresponding oxygenated xanthophylls are below detection limits. Therefore, the search for mutants in which those precursors accumulate has been undertaken for many years.[1] With new developments in the field of molecular genetics, carotenoid mutants have become available by direct manipulation of appropriate genes, for example, by transposon mutagenesis[2] or by deletion restriction of gene fragments.[3] Nevertheless, conventional mutagenesis is still very helpful and provides specific mutants which have not yet been obtained in other ways. Two examples will be presented in this chapter. One is *Escherichia coli* transformed with a carotenogenic gene cluster from *Erwinia herbicola*.[4] Mutants have been generated by partial digestion of the genes involved in carotenogenesis.[3] Analysis by HPLC showed formation of zeaxanthin diglycoside in the transformant and various intermediates in these mutants. The other organism is the green alga *Scenedesmus obliquus*. Ultraviolet mutagenesis resulted in the mutant C-6D[5] which under heterotrophic conditions forms untypical poly-*cis*-carotenes.[6]

Characterization of Mutants

Cultivation of Mutants

From the genomic library of *Erwinia herbicola* a 12.4-kilobase (kb) fragment containing all the genes necessary for carotenoid formation has been cloned.[4,7] Growth of the transformed *E. coli* LE392/pPL376 is carried

[1] P. M. Bramley and A. Mackenzie, *Curr. Top. Cell. Regul.* **29,** 291 (1988).
[2] K. M. Zsebo and J. E. Hearst, *Cell (Cambridge, Mass.)* **37,** 937 (1984).
[3] G. Schnurr, A. Schmidt, and G. Sandmann, *FEMS Microb. Lett.* **78,** 157 (1991).
[4] K. L. Perry, T. A. Simonitch, K. J. Harrison-Lavoie, and S. T. Liu, *J. Bacteriol.* **168,** 607 (1986).
[5] N. I. Bishop, *in* "Methods in Chloroplast Molecular Biology" (M. Edelmann *et al.*, eds.), p. 51. Elsevier Biomedical, Amsterdam, 1982.
[6] S. Ernst and G. Sandmann, *Arch. Microbiol.* **150,** 590 (1988).
[7] G. Sandmann, W. S. Woods, and R. W. Tuveson, *FEMS Microb. Lett.* **71,** 77 (1990).

out in Luria–Bertani (LB) liquid medium containing 10 g/liter Bactotryptone (Difco), 5 g/liter yeast extract, and 10 g/liter NaCl, adjusted to pH 7.5. As the plasmid confers ampicillin resistance, 75 μg/ml of this antibiotic is added. After inoculation, the bacterial cultures are incubated at 30–35° with continuous shaking.

The C-6D mutant of *Scenedesmus obliquus* was provided by Prof. N. Bishop (University of Corvallis, OR). It is grown as described[8] in complete darkness under heterotrophic conditions. Therefore, the liquid cultures are supplemented with 5 g/liter glucose and 2.5 g/liter yeast extract and grown at 20° on a shaker, with a stream of air being bubbled through. Cells are harvested after 7 days.

Extraction of Carotenoids

All extraction steps should be carried out under dim light to avoid photodegradation and isomerization. Lyophilized cells (20 mg) are suspended in 20 ml of methanol, and 2 ml of 60% KOH (w/v) is added. Carotenoids are extracted at 65° in darkness for 20 min. Then the extract is poured into 15 ml of diethyl ether in a separatory funnel, and 10 ml of saturated NaCl solution is added. After partitioning, the upper diethyl ether layer is collected and the partitioning repeated with the lower phase. The combined diethyl ether extract is dried over anhydrous Na_2SO_4, and the solvent is evaporated under reduced pressure. The residue can be stored under nitrogen in a sealed vessel in a freezer or directly dissolved in 100 μl of methanol for further use.

Chromatographic Separation, Photoisomerization, and Reference Compounds

For analysis of carotenoids, the isocratic HPLC system described by Märki-Fischer *et al.*[9] is employed. It is suitable for separation of various carotenes, including their cis/trans isomers, and of different xanthophylls. The column (25 cm) is filled with Spherisorb ODS-1 5 μm, the eluent consists of acetonitrile/methanol/2-propanol (85:10:5, v/v), and the flow rate is 1 ml/min. Absorbances at different wavelengths are measured with a Waters 994 programmable photodiode array detector, and spectra are recorded from the eluent peaks.

Poly-*cis*-carotenes can be converted to their all-*trans* forms by iodine isomerization. The carotenes are dissolved in 2 ml of hexane to give an absorbance at the wavelength of the central spectral maximum of 1.5. This corresponds to a total amount of 10 to 15 μg. Then 10 μl of an iodine

[8] G. Sandmann and P. Böger, *Photosynth. Res.* **2**, 281 (1981).

[9] E. Märki-Fischer, U. Marti, R. Buchecker, and C. H. Eugster, *Helv. Chim. Acta* **66**, 494 (1983).

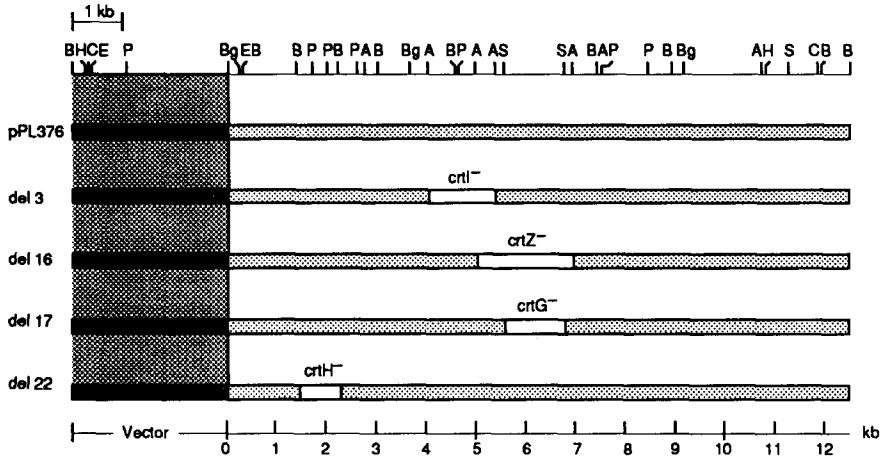

FIG. 1. Deletion mutants in a carotenogenic gene cluster of transformant *E. coli* LE392/pPL376.

solution in hexane (30 μg/ml) is added, and the test tube is illuminated for 30 min with an Osram L40/W25 fluorescence lamp at a distance of 20 cm.

The following reference carotenoids are employed for HPLC: all-*trans*-carotene, neurosporene, and lycopene are isolated from fungal mutants,[6] and pro-ζ-carotene, proneurosporene, and prolycopene from the tomato variety Tangella.[10] 15-*cis*-Phytoene is extracted from cress seedlings germinated for 3 days on filter paper in the dark in water containing 0.5 μM of the bleaching herbicide norflurazon (trade names: Zorial, Evital, or Solican). Zeaxanthin and β-cryptoxanthin are isolated from the cyanobacterium *Aphanocapsa* PCC 6714.[11] Isolation procedures are similar to the one used for *Scenedesmus* and *E. coli,* and purification is performed by two subsequent TLC steps. Carotenes are first separated on silica gel plates with 15% toluene in hexane and then on activated Al_2O_3 plates with 0.7% acetone in hexane. The two subsequent TLC systems for xanthophyll purification are silica plates with toluene/ethyl acetate/methanol (75:20:5, v/v) and then Al_2O_3 plates with 35% acetone in hexane.

Carotenoids of Deletion Mutants from Escherichia coli LE392/pPL376

Deletions in pPL376 are obtained by partial digestion of plasmid DNA with different restriction enzymes and directional cloning of restriction fragments. The details are given elsewhere.[3] Figure 1 shows the 12.4-kb

[10] J. M. Clough and G. Pattenden, *J. Chem. Soc. Chem. Commun.*, 616 (1979).
[11] P. M. Bramley and G. Sandmann, *Phytochemistry* **24**, 2919 (1985).

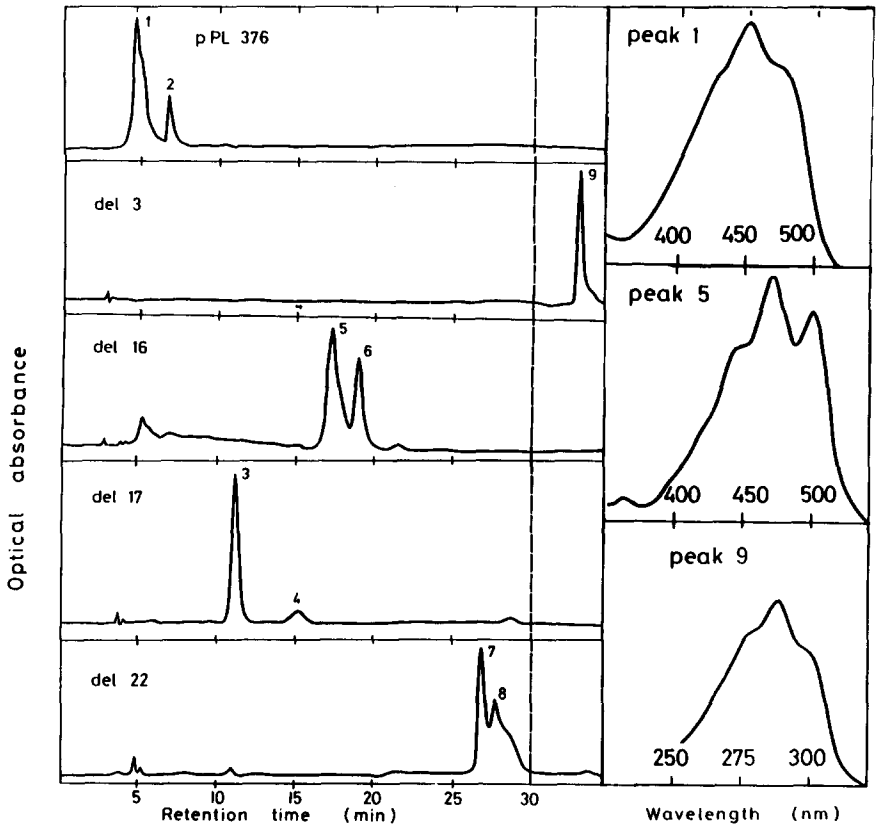

FIG. 2. HPLC analysis and absorbance spectra of carotenoids accumulating in different deletion mutants of *E. coli* LE392/pPL376.

gene cluster from *Erwinia herbicola* which contains six genes encoding the carotenoid pathway to zeaxanthin diglycoside.[3] Deletions in the genes *crtI, crtZ, crtH,* and *crtG* have been generated. The resulting mutants *del 3, 16, 17,* and *22* accumulate different carotenoid intermediates.

Depending on the growth conditions, *E. coli* LE392/pPL376 accumulates large amounts of colored carotenoids. The dominant pigments (>90%) in *Erwinia herbicola* are zeaxanthin mono- and diglycoside,[7] which are the same carotenogenic end products as in *Erwinia uredevora.*[12] These highly polar glycosides chromatograph at 5 (diglycoside) and 7 min

[12] N. Misawa, M. Nakagawa, K. Kobayashi, S. Yamano, Y. Izawa, K. Nakamura, and K. Harashima, *J. Bacteriol.* **172,** 6704 (1990).

(monoglycoside) in the reversed-phase HPLC system used in Fig. 2. All deletion mutants have been analyzed for carotenoid content by HPLC with corresponding standards for all the carotenoids found. The deletion mutant *del 3* did not accumulate any colored carotenoid. Instead, 15-*cis*-phytoene was found at R_t 33.1 min (peak 9, Fig. 2). It showed its typical UV spectrum with absorbance maxima at 276, 287, and 298 nm. Two carotenes with a lycopene-like spectrum were found in strain *del 16* at R_t 16.8 (absorbance maxima at 446, 471, and 502 nm) and 18.5 min (peaks 5 and 6, Fig. 2). The fine structure in the region between 330 and 380 nm is typical for an all-trans isomer of lycopene in case of peak 5 (Fig. 2) and for a mono-cis configuration of one of the central double bonds in peak 6 (Fig. 2). In *del 17* the major peak (peak 3, Fig. 2) consisted of zeaxanthin (R_t 10.8 min). In addition, small amounts of its monohydroxylated precursor β-cryptoxanthin (peak 4, Fig. 2; R_t 15.5 min) were present; *del 22* accumulated only nonhydroxylated all-*trans*-β-carotene (peak 7, Fig. 2; R_t 26.9 min) and a cis isomer (peak 8, Fig. 2; at 27.7 min).

The carotenoid pattern in these deletion mutants indicates that in *del 3* pytoene desaturase encoded by the gene *crtI* is inactivated. In *del 16*

TABLE I

ABSORBANCE MAXIMA AND RETENTION TIMES OF all-*trans* AND POLY-*cis*-CAROTENES FROM *Scenedesmus* C-6D

Carotenes	Absorbance maximum (nm) in eluent for HPLC			R_t (min)
A. Reference compounds for all-*trans* pathways				
Lycopene (all-*trans*)	440	471	498	14.67
Neurosporene (all-*trans*)	416	439	468	15.33
γ-Carotene (all-*trans*)	440	460	490	16.97
ζ-Carotene (all-*trans*)	382	402	427	18.40
α-Carotene (all-*trans*)	421	445	472	21.92
β-Carotene (all-*trans*)	425	452	477	22.82
Phytofluene (all-*trans*)	332	349	368	25.00
Phytoene (15-*cis*)	277	286	298	27.83
B. Carotenes from *Scenedesmus* C-6D				
Prolycopene (7,9,7′,9′-poly-*cis*)	418	438	460	16.85
Proneurosporene (9,7′,9′-poly-*cis*)	408	432	459	18.65
cis-ζ-Carotene II (?-*cis*)	382	402	427	19.00
Pro-ζ-carotene (9,9′-di-*cis*)	382	402	427	20.55
Prophytofluene (9,15-di-*cis*)	332	349	368	25.00
Phytofluene II (?-*cis*)	332	349	368	25.00
Phytoene (15-*cis*)	277	286	298	27.83

lycopene cyclization (gene *crtZ*) is impaired, and *del 17* with a modified *crtH* gene lacks expression of β-carotene hydroxylase. Finally, in mutant *del 22* the glycosylation step (gene *crtH*) is missing.

Poly-cis-carotenes from Scenedesmus C-6D

In *Scenedesmus* C-6D only acyclic carotenes are present. None of them exhibits the usual all-trans configuration which is typical for colored carotenoids. The HPLC retention times of all-*trans*-carotenes which normally participate in the biosynthetic pathway are shown in Table I together with their absorbance maxima. The only carotene from C-6D also formed by wild-type *Scenedesmus* or other photosynthetic organisms is 15-*cis*-phytoene.

Aside from phytofluene II and ζ-carotene II, the isomeric configurations could be assigned as the ones known from the series of procarotenes found in the tomato mutant Tangella.[11] By cochromatography with carotenes from this fruit, prophytofluene, pro-ζ-carotene, proneurosporene, and prolycopene can be identified. In addition, proneurosporene and prolycopene show absorbance spectra which are different from those of the corresponding all-trans isomers. For the separation of phytofluene isomers the HPLC solvent system is modified to acetonitrile/methanol/2-propanol (95:3:2, v/v) at a flow rate of 0.6 ml/min.[10]

An additional possibility to identify *cis*-carotenoids is by iodine-catalyzed photoisomerization to all-trans and other cis isomers. Then, changes in their spectral properties and separation characteristics are observed. The isomerization conditions employed cause the complete conversion of poly-*cis*-carotenes to several other cis isomers or into all-trans forms. These isomers are formed from pro-ζ-carotene (Fig. 3A). ζ-Carotene I runs with the same retention time on HPLC as pro-ζ-carotene. However, in contrast to pro-ζ-carotene, the spectrum of ζ-carotene I exhibits a pronounced cis peak at 297 nm and a different ratio of the two main absorbance maxima. Both maxima show a shift of 3 nm to lower wavelengths compared to pro-ζ-carotene. ζ-Carotene II is the same cis isomer already specified in Table I. At the shortest retention time of all-ζ-carotene isomers, all-*trans*-ζ-carotene ($t - \zeta C$) can be identified with a standard and by its spectrum. In case of proneurosporenes this isomer and all the others obtained after isomerization can be well separated from each other by HPLC (Fig. 3B). They also differ by the position of the main absorbance maximum. The major isomerization products are two different cis isomers, whereas all-*trans*-neurosporene is found to a lesser extent. The spectrum of prolycopene shows the main absorbance peak at the lowest wavelengths of all lycopene isomers. After isomerization no significant

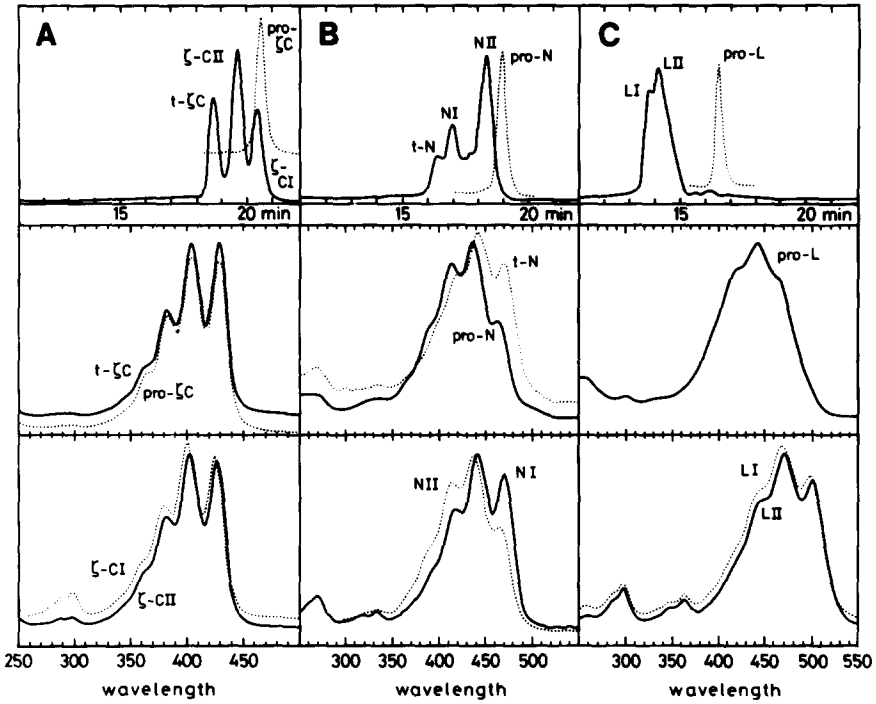

FIG. 3. HPLC separation of photoisomerization products of pro-ζ-carotene (A), proneurosporene (B), and prolycopene (C).

amounts of all-*trans*-lycopene could be detected by cochromatography (Fig. 3C). The spectra of the two lycopene isomers found are those of cis isomers as indicated by their cis peaks at 362 nm and a wavelength shift of the main absorbance maximum.

Acknowledgments

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